CHARACTERIZATION OF PROTEIN SECRETIONS BY THE PORCINE UTERUS AND THEIR RELATIONSHIP TO REPRODUCTIVE PHYSIOLOGY

By

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The uterus actively influences reproduction in many species of mammals by participation in embryonic differentiation and in determination of estrous cycle length. In this work the secretion of proteins by the porcine uterus during the estrous cycle was characterized and related to reproductive phenomena.

To determine if the porcine uterus provides an environment essential for continuous development of early embryos, an experiment which involved restriction of embryos to the oviduct was carried out. One tubo-uterine junction was ligated in each of 10 mated gilts under surgical anesthesia at 48 to 72 hours post coitum. The contralateral uterine horn was ligated approximately 10 cm posterior to the tubo-uterine junction and served as a control. The animals were slaughtered either 7 or 8 days after mating and the embryos were recovered, examined, and measured. The tube-locked embryos failed to develop past the morula stage and many had begun degenerating by the time of recovery. No tube-locked embryo had escaped from the zona pellucida. However,

uterine embryos in the same females were mostly blastocysts, and 84 percent had emerged from the zona pellucida and formed a distinct inner cell mass. These data suggest that some factor secreted by the porcine uterus is required by embryos for continued development.

Daily quantitative and qualitative variation in the protein constituents of uterine intraluminal fluid was studied on days 2 to 18 and day 20 of the estrous cycle. Sixty-six gilts furnished one sample of uterine fluid each. Samples were centrifuged at 3000 g, sterilized by millipore filtration, concentrated from approximately 40 ml to 1 ml, and dialyzed against 0.05 M phosphate-citrate buffer, pH 7.4. Gel filtration through Sephadex G-200 was used to establish protein profiles for each sample and for estimation of molecular weights. Polyacrylamide gel disc electrophoresis was employed to compare the protein components among the various samples.

Gel filtration revealed three groups of proteins (Fractions I, II, III) which did not vary with the estrous cycle and two groups of proteins (Fractions IV and V) which were not present early or late in the estrous cycle. Fraction IV, which appeared on day 12, contained a lavender protein with an estimated molecular weight of 45,000, while Fraction V had an estimated molecular weight of about 20,000, and appeared as early as day 9. Fractions IV and V were not present in the gel filtration protein profiles after day 16 of the cycle.

Disc electrophoresis demonstrated that Fraction IV contained at least three basic proteins, while Fraction V contained one basic protein and six acidic proteins at pH 8.0. The basic proteins, especially, are interesting as possible regulators of embryonic differentiation by control of genetic expression. The fact that these proteins appear in

the uterine flushings shortly before the corpus luteum begins to regress and are not present after regression suggests an interrelationship with the corpus luteum.

Attempts to determine the role of the uterine fluid proteins in reproductive phenomena were largely unsuccessful. Anti-uterine fluid sera did not interfere with pregnancy when injected intravenously into mated gilts. Final conclusions cannot be drawn from these data since it is possible that the antibodies injected did not reach the uterine lumen in sufficient quantities to bind proteins secreted by the uterus.

This study showed that the uterine environment is required for embryonic development and that there are cyclic changes in the protein secretion pattern of the uterus. These findings provide additional support for the concept that proteins of maternal origin may regulate genetic expression in the embryo during early differentiation.

INTRODUCTION

The mammalian uterus serves in the reproductive process to protect the embryo, to supply nutrition to the embryo, and to participate in the differentiation of the embryo. The latter function is the least understood, but perhaps the most important. Evidence has accumulated that some factor provided by the uterine environment is required for uninterrupted embryonic development and that the uterus is actively involved in the reproductive process.

The active role of the uterus in reproduction has been studied in a variety of experiments, using indirect methods such as ova culture, superovulation, embryo superinduction, and hysterectomy. Using a direct approach Krishnan and Daniel (1967) identified, in rabbit uterine fluid, a protein which stimulates blastulation of rabbit embryos *in vitro*. This is the first uterine protein demonstrated to have an apparent regulatory function in embryonic development. This information is extremely important because it provides clues into the mechanism by which the uterus exerts an active control over embryonic development.

There is also direct evidence that the uterus actively participates in the regulation of the life span of the corpus luteum. The evidence suggests that this regulatory factor is also proteinic in character. If uterine proteins with regulatory functions on embryonic development and the corpus luteum are a general rule in mammalian reproduction, methods for either increasing fertility or decreasing it to zero may be near at hand. Also, interesting possibilities for study

of the mechanism of differentiation of mammalian cells as influenced by specific proteins are apparent.

Considering the available information on these roles of the uterus, a working hypothesis can be formulated concerning the regulatory function of the uterus in reproduction. The working hypothesis, which served as a basis for these studies can be stated as follows: the uterus secretes a protein(s) which is required by the embryo for uninterrupted development; but, if embryos are not present to utilize the protein(s), it causes regression of the corpus luteum.

This study was carried out to determine if the uterine environment is required for embryonic development in the pig and to qualitatively and quantitatively characterize the secretion of protein by the porcine uterus during the estrous cycle. In addition, attempts were made to relate the proteins secreted by the uterus to embryonic development.

CHAPTER 1. REVIEW OF LITERATURE

In recent years research has made it clear that the uterus plays a much more profound role in mammalian reproduction than the rather passive functions of nutrition and protection of the embryo. There is much evidence that the uterus influences embryonic development in a very specific way and that the life span of the corpus luteum is under the control of the uterus. These active roles of the uterus are suggested by several lines of evidence as will be discussed below.

One line of evidence was developed from experiments with *in vitro* culture of the embryos. Lewis and Gregory (1929) found that rabbit ova developed in rabbit blood plasma to the blastocyst stage but no further. This failure of development past the early blastocyst stage *in vitro* has been the general rule regardless of the composition of the culture medium or the species of animal studied (Chang, 1949; Hammond, 1949; Tarkowski, 1961; Brinster, 1963; Adams, 1965; Cole and Paul, 1965; Onuma *et al.*, 1968; Maurer *et al.*, 1969; and Rundell, 1969). Recently two groups of workers (Whitten and Biggers, 1968; and Kane and Foote, 1970 a,b,c) have been somewhat more successful in embryo culture and have grown embryos to the beginning of blastocyst expansion in chemically defined media alone or in media containing bovine serum fractions. However, continued development of preblastocyst embryos past the early blastocyst stage *in vitro* has not been reported.

In vitro culture of more advanced embryos has lately been achieved by use of a device developed by New (1967) for recirculating

the culture medium. New (1967) reported that 5- to 10-somite rat embryos grew to 30 to 35 somites, and 25-somite embryos grew to 40 to 45 somites in recirculating homologous serum. Embryos more advanced than 45 somites did not develop further *in vitro*. Using the New (1967) recirculator or modifications of it, hamster embryos have been grown to a stage of development at which 50 percent of the embryos had limb buds and 63 percent had good circulation of blood (Givelber and DiPaolo, 1968), rat embryos explanted at 7.5 days *post coitum* have been grown to the 15- to 20-somite stage (New and Daniel, 1969), and rabbit embryos explanted at 6.8 days *post coitum* have been cultured to the 23-somite stage (Daniel, 1970a). Thus after achieving the blastocyst stage *in utero* and implanting, embryos become at least temporarily independent of the uterine environment.

Other evidence for an active role of the uterus in embryonic development is derived from research which indicates that the oviduct can support development to the morula or early blastocyst stage, but no further. Restriction of embryos to the oviducal environment by ligation of the tubo-uterine junction does not prevent development to the early blastocyst stage in mice (Kirby, 1962; and Orsini and McLaren, 1967), rabbits (Westman *et al.*, 1931; Pincus and Kirsch, 1936; and Adams, 1958), rats (Alden, 1942), and sheep (Wintenberger-Torres, 1956); however, further development of tube-locked embryos has not been observed. Adams (1958), Kirby (1962), and Orsini and McLaren (1967) reported that the tube-locked embryos not only failed to continue development, but underwent degeneration within the oviduct.

Transplantation of rat and mouse tubal embryos to extrauterine sites has been attempted, but with little apparent success. Fawcett *et al.* (1947) and Runner (1947) transplanted mouse oviducal embryos to the anterior chamber of the eye. Implantation occurred but only extraembryonic

membranes developed. Similar abnormal growth was observed in mouse tubal embryos (Fawcett, 1950; and Kirby, 1962) and in rat tubal embryos (Nicholas, 1942) transplanted beneath the kidney capsule. However, developmental failure of extrauterine embryos is not a universal phenomenon. It is common knowledge that extrauterine embryonic development occurs in at least one species, i.e., the human, where ectopic pregnancies in a variety of extrauterine sites have been reported.

Experiments involving superovulation and embryo superinduction have provided still further evidence for the role of the uterus in control of early embryonic development. Bazer et al. (1969a) reported that litter size in swine at 90 days post coitum was similar for unoperated controls, sham-operated controls, and for three levels of embryo superinduction. The number of corpora lutea in each recipient was counted and considered to represent the number of native embryos. Embryo superinduction was achieved by transfer of additional 2- to 4-cell embryos into the uteri of pregnant recipients. The three groups receiving extra embryos had 16, 22, or 28 potential embryos after transfer. At 90 days post coitum average litter size in the five groups was 9.9, 8.2, 8.8, 8.5, and 9.9 for the control, sham, 16-embryo, 22-embryo, and 28-embryo groups respectively. The authors referred to this limitation in litter size as "uterine capacity."

In the same experiment, Bazer et al. (1969a) observed that synchrony of transferred embryos and recipient uteri is very important. When development of foreign (transferred) embryos relative to native embryos was equal, the survival of native embryos was 51 percent compared to 35 percent for foreign embryos and this difference was not significant. When the foreign embryos were 12 hours behind the native embryos, the

survival rates were 56 percent for native embryos compared to 20 percent for foreign; and, even more strikingly, when foreign embryos were 24 hours behind the native embryos, survival rates were 72 percent for native compared to 3 percent for foreign embryos. These differences in survival rates were highly significant, and they indicate that embryos in synchrony with the uterus are at a great advantage in competing for possible uterine factors as compared to asynchronous embryos. This suggests that embryos must reach a precise stage of development before they can utilize the uterine factor and that the uterine factor is available only at a specific time. Kirby (1962) found this to be the case in the mouse. When 3.5-day tube-locked blastocysts were transferred to the uterus, development continued normally, but if the blastocysts were forced to remain in the oviduct until 4.5 days post coitum, further development did not occur after transfer to the uterus. Other authors have also reported that synchronization of donors and recipients is necessary for survival of transferred ova in the rat (Dickmann and Noyes, 1960) and in the pig (Hunter et al., 1967). However Webel et al. (1970) reported that asynchrony by plus or minus 1 day did not affect embryo survival in swine.

Bazer et al. (1969b) compared embryonic survival after embryo superinduction at 25 days and at 105 days post coitum. Among gilts slaughtered at 25 days post coitum the average number of potential embryos (corpora lutea plus transferred embryos) was 23.9, and the average litter size was 12.0 (50 percent survival). In the group of gilts slaughtered at 105 days, there were 23.6 potential embryos and the average litter size was 9.8 (42 percent survival). Unoperated control gilts had litter sizes of 9.6 at 25 days and at 105 days post coitum, with 74 and 75 percent survival, respectively. Thus this experiment shows clearly that the

majority of embryonic deaths following embryo superinduction occur prior to 25 days post coitum. This is in agreement with data from untreated gilts (Gossett and Sorenson, 1959).

Fenton et al. (1969a) reported that embryo superinduction of gilts by transfer of 7-day blastocysts resulted in a significant increase in average litter size at 25 days after mating, as compared to litter size in control gilts. Therefore, it was postulated that "uterine capacity" is established prior to day 7. To test this hypothesis, Fenton et al. (1969b) used 2.5-day and 7-day embryos and Schwartz et al. (1970) used 7-day embryos in embryo superinduction of gilts. Average litter sizes at 25 days post coitum were not different from those in control gilts; therefore, contrary to the results of Fenton et al. (1969a), it appears that "uterine capacity" is not established prior to day 7, but instead between day 7 and day 25.

The effects of restricted uterine space on embryonic development has also been studied. In swine unilateral hysterectomy-ovariectomy (Dziuk, 1968; and Fenton et al., 1970) did not affect litter size at 25 days after mating. Variation of the amount of uterine space by ligation of the uterine horns so that embryos on one side had half the uterine space available to embryos in the contralateral horn (Dziuk, 1968) did not affect embryonic survival in swine. In mice, rabbits, and rats Johnson (1970) established pregnancy in one of the two uterine horns by unilateral ovariectomy. In these animals, ovulation rate was similar to that in intact females, and litter size was no different even though the amount of uterine space was only half that available to embryos in intact females. Similar results have been reported earlier by Hafez (1964) using rabbits. Thus in mice, rabbits, and rats one uterine horn can support as many embryos as two uterine horns. However, final litter size at

105 days after mating was decreased by surgical reduction in uterine tissue in the pig (Fenton et al., 1970).

Superovulation is followed by increased embryonic death in swine so that litter size at farrowing is not significantly greater than in control gilts (Hammond, 1921; Perry, 1954; Rathnasabapathy et al., 1957; King and Young, 1957; Hunter, 1966; Dziuk, 1968; Longenecker and Day, 1968; Pope et al., 1968; and Bazer et al., 1969c). Also, in rabbits superovulation does not increase litter size and even tends to decrease the number of feti surviving to term. Adams (1960) reported that superovulation resulted in a mean of 28.7 implantations but only 6.5 surviving feti at term in rabbits. Hafez (1964) also found that superovulation failed to increase litter size in rabbits and that the maximum number of viable feti at term was 8 per uterine horn and 15 per litter. Johnson (1970) reported that in rabbits average litter size could not be increased significantly, but that one uterine horn could carry as many embryos as two horns. In mice the litter size is increased following superovulation (McLaren and Michie, 1956; McLaren and Michie, 1959 a,b; and Wilson and Edwards, 1963). On the other hand, Fowler and Edwards (1957) were unable to increase litter size by superovulation of mice. In general, polytocous species appear to have a limit to the number of embryos that can be maintained to term and in pigs and rabbits this limit is apparently closely approached in the normal pregnancy. Mice seem to be an exception to this general rule, suggesting that the number of ova shed is less than the number required to reach the "uterine capacity" of this species.

Further evidence for uterine control of the fate of blastocysts is found in the research on delayed implantation of embryos. As indicated

in a review of this subject by Daniel (1970b), delayed implantation occurs in a wide variety of species. In some species the delay is obligative while in others it is facultative (occurring only when the female becomes pregnant during lactation). In rats and mice implantation can be prevented without degeneration of the blastocysts by ovariectomy prior to day 4 post coitum and daily treatment with 2 mg progesterone (Dickmann, 1967). Implantation can be induced in such animals by a single 1 µg dose of estrone. On the other hand, Humphrey (1969) did not need to administer estrogen to ovariectomized, progesterone-treated mice in order to cause implantation.

The role of the uterus in implantation and the importance of the hormonal state of the female in this role was clearly demonstrated by Dickmann and DeFeo (1967). In this experiment rats were ovariectomized on the second day of pregnancy in order to provide uteri and blastocysts in the delayed state, i.e., dormant. A group of intact female rats served as a source of active uteri and blastocysts. When dormant embryos were transplanted to the active uteri, they became active and implanted within a few hours after being placed in the active uteri. When active blastocysts were transplanted to dormant uteri, these embryos became dormant and remained dormant until implantation was induced by administering estrogen to the female. Thus it is the uterus which determines the condition of the embryos.

Experiments on delayed implantation in general and, more specifically, the work of Dickmann and DeFeo (1967) indicate that progesterone and estrogen are very important to the role of the uterus in controlling embryonic development. The specificity of the uterus as a target organ for estrogen has been clearly shown by Gorski and co-workers (1968). In a series of experiments,

these authors found that estrogenic compounds were specifically bound to receptor molecules in the rat uterus. One receptor was a 9.5 S protein component in the uterine cytosol and the other was a 5 S protein in the nucleus. Nonestrogenic steroids had no apparent interaction with the receptor molecules. Jensen and DeSombre (1969) showed that the uterine uptake of estrogen is unsaturable even with hyperphysiological levels of estrogen; however, the retention of estrogen is limited.

Estrogens have been shown to induce nuclear RNA synthesis and uptake of RNA precursors in the uterus within two minutes after injection (Means and Hamilton, 1967). Hamilton et al. (1967) reported that the rate of nuclear RNA synthesis and activity of Mg^{++}-activated RNA polymerase was greater at estrus than at diestrus; whereas, nuclear RNA and protein content did not vary with the estrous cycle. Whole uterine tissue content of both RNA and protein per cell was two and five times greater, respectively, at estrus than during diestrus. Contrary to the work of Hamilton and co-workers, Billing et al. (1969) reported that uterine RNA synthesis, as indicated by incorporation of radioisotope-labeled precursors, did not increase until five hours after estradiol treatment and that the RNA synthesized was mainly r-RNA and t-RNA with very little m-RNA. From these experiments it is evident that estrogenic compounds profoundly influence the metabolism of the uterus, inducing both RNA and protein synthesis. Notides and Gorski (1966) reported that estradiol stimulated synthesis of a new protein specific to the rat uterus both in vivo and in vitro. This protein appeared within 30 minutes after administration of estrogen, but contributed only a minor amount to the quantity of total protein in the uterus.

There are few reports in the literature which suggest induction of RNA and protein synthesis by progesterone. However there is ample evidence for induction of at least one specific protein by progesterone. Hertz et al. (1943) showed that progesterone induced the biosynthesis of avidin in the estrogen pretreated oviduct of the immature chick. O'Malley (1967) found that progesterone could induce avidin synthesis in minced chick oviduct tissue in vitro. Dingman et al. (1969) reported that progesterone stimulated biosynthesis of both nuclear and cytoplasmic t-RNA in the chick oviduct. It has been shown that progesterone induces synthesis of new species of m-RNA by the chick oviduct prior to the initiation of avidin biosynthesis (O'Malley and McGuire, 1969). Aside from its effect on avidin biosynthesis, progesterone reportedly has a detrimental influence on estrogen-induced RNA synthesis in the rat uterus (Trams and Maass, 1969) and in rat mammary tissue (Eddington, 1969). The meager amount of information available suggests that any role of progesterone in RNA and protein biosynthesis is likely to be highly specific.

The protein constituents of uterine luminal fluids have been investigated in a number of studies in recent years. Junge and Blandau (1958) found four major electrophoretic components present at low levels in rat uterine fluids. Albumin was usually not present in the uterine fluid obtained by these authors. Ringler (1961) reported that rat uterine fluid contained proteins with five different electrophoretic mobilities. Of these five components, a prealbumin fraction was specific to uterine fluid. Ringler proposed that uterine luminal fluid is essentially an ultrafiltrate of plasma but includes specific uterine secretions. Utilizing the techniques of Öuchterlony gel diffusion and

immunoelectrophoresis, Albers and Castro (1961) showed that pooled and concentrated rat uterine fluid contains at least five protein components. Only one of the five components was specific to uterine fluid, and it migrated to a position occupied by β-globulins in serum.

The proteins of rabbit uterine fluid were first studied by Stevens et al. (1964). These workers used diffusion-in-gel, moving boundary electrophoresis, and immunoelectrophoresis to characterize the proteins in fluids from the ligated uteri of estrous rabbits. Moving boundary electrophoresis separated eight components, among which a prealbumin and an α-globulin were specific to uterine fluid and not found in serum. Diffusion of uterine fluid in gel revealed 13 antigenic components of which three were unique to uterine fluid. Five antigens specific to uterine fluid were made evident by immunoelectrophoresis. Two of the five antigens had mobilities in the prealbumin region and three were similar to β-globulins.

In 1967, Krishnan and Daniel studied the uterine fluid protein constituents of rabbits in early pregnancy. The fluid was subjected to gel filtration through Sephadex G-200 and five major protein fractions were obtained. The fourth fraction was not present in the chromatographic profiles until day 3 post coitum. The maximum concentration of this fraction occurred on day 5 post coitum and afterward appeared in progressively smaller proportions of the total protein until day 9. The variable fraction was also observed in uterine fluid of pseudopregnant rabbits on day 7 post coitum and blastocoelic fluid, but not in uterine fluid accumulated by ligation of the rabbit uterus from day 3 to day 10 of pregnancy, maternal serum, fetal serum, or fetal amniotic fluid. This fraction was used as a supplement to Ham's F 10 culture medium in rabbit

embryo culture and it promoted the development of embryos to the expanding blastocyst stage. This property prompted the authors to call this fraction "blastokinin." The molecular weight of "blastokinin" was estimated by gel filtration to be 27,000, and it was found to be a glycoprotein. In a later paper (Krishnan and Daniel, 1968) it was reported that amino acids constituted about 74 percent of the protein and 6 percent was contributed by carbohydrates. No sialic acid was found in the glycoprotein.

Beier (1968) reported that, based on immunologic and electrophoretic techniques, flushed uterine fluid and blastocoelic fluid obtained on the sixth day of pregnancy contained plasma proteins as well as specific uterine fluid proteins. One of the proteins specific to the uterus was reported to be involved in blastocyst development and was designated "uteroglobin." "Uteroglobin" was shown to be a glycoprotein with a molecular weight of about 30,000 as determined by ultracentrifugation. Steroid hormones were shown to have a profound effect on the level of "uteroglobin" in 6-day post coitum uterine fluid. Treatment of rabbits with estradiol for three days (100, 100, and 50 µg) plus 5 mg progesterone per day for five days caused the relative percent of "uteroglobin" to be double that in the normal 6-day pregnant rabbit. Administration of excessive amounts of estrogen caused a reduction in the amount of "uteroglobin" produced. Kirchner (1969) also reported a specific glycoprotein in rabbit uterine secretion. Hamana and Hafez (1970) demonstrated "uteroglobin" in rabbit blastocoelic fluid between days 3 and 9 of pregnancy. Both Kirchner (1969) and Beier (1970) believed "uteroglobin" and "blastokinin" to be the same protein.

As was discussed earlier in this literature review, it is a general rule that embryos can develop no further than the morula or early blastocyst stage in synthetic media *in vitro*. There have been exceptions to this rule (Whitten and Biggers, 1968; and Kane and Foote, 1970 a,b,c); however, this does not diminish the significance of "blastokinin" since the effect of the presence and absence of "blastokinin" were observed under the same experimental conditions. The stimulatory effect of "blastokinin" on rabbit embryos *in vitro* suggests that it may be involved in the process of implantation (Beier, 1970).

Daniel (1968 and 1970 b) and Daniel and Krishnan (1969) have studied the relationship between uterine fluid proteins and embryonic diapause in a number of species of mammals having delayed implantation. Daniel (1968) obtained fluid from the rat, during delayed implantation and in normal pregnancy; the fur seal; the armadillo; and the mink. Polyacrylamide gel disc electrophoresis revealed no protein with similar electrophoretic mobility to "blastokinin" except possibly in low concentration in uterine fluid of mink.

Daniel and Krishnan (1969) found that the protein content of uterine flushings of animals undergoing delayed implantation was much lower than was the case in other species. These authors found a small "blastokinin"-like fraction in the Sephadex gel filtration profiles of mink uterine fluid obtained on day 20 *post coitum*, but not prior to this time. This is just before the time at which mink embryos implant (Baevsky, 1963). Diapausing embryos of animals with obligative delayed implantation (mink, fur seal, and armadillo) were stimulated to grow *in vitro* in the presence of rabbit "blastokinin" as indicated by mitotic activity but not in media containing serum. Diapausing rat blastocysts

(i.e., species with facultative delayed implantation) expanded *in vitro* in Ham's F 10 medium alone, in medium containing maternal serum, or in medium containing macromolecular components of uterine secretion, but not rabbit blastokinin. Thus, in facultative delayed implantation there is apparently not only a protein deficiency but also some other uterine condition which inhibits growth of the blastocysts.

The role of the uterus is not limited solely to control of embryonic development but it also affects life span of the corpus luteum in at least some animals (see reviews by Anderson *et al.*, 1963; Bland and Donovan, 1966; Anderson *et al.*, 1969; Schomberg, 1969; and Rowson, 1970). In these reviews it was pointed out that hysterectomy in cattle, pigs, sheep, and other species results in maintenance of the corpus luteum for approximately the length of normal gestation. Goding *et al.* (1967) showed that if the uterus of a sheep was transplanted to the neck, while leaving the ovaries intact, estrous cycle lengths were increased. Harrison *et al.* (1968) showed that if both the ovary and the uterus were transplanted together to the neck the cycle lengths were normal. These experiments suggest that the luteolytic factor is not mediated via the pituitary gland or nervous connections.

Caldwell *et al.* (1967) reported that homologous uterine transplantation into the cheek pouches of the Syrian hamster caused regression of the corpora lutea in hysterectomized hamsters. Transplantation of uteri from estrous rats, uteri from 14-day pseudopregnant rabbits, and homologous endometrium into the cheek pouch of the hysterectomized Syrian hamster caused regression of corpora lutea. Similar transplantations of human endometrium and homologous myometrium were ineffective. Caldwell *et al.* (1968) reported that when transplanted

to the neck of hysterectomized pseudopregnant hamsters, sheep endometrium obtained toward the end of the cycle caused regression of the corpora lutea. This suggests that an endometrial secretion that is not species specific is responsible for luteolysis.

Several early attempts to demonstrate luteolytic effects of endometrial extracts were largely unsuccessful (see Schomberg, 1969) until Williams et al. (1967 a,b) reported that acetone-dried extracts of bovine uteri caused luteal regression in the rabbit. Schomberg (1967) applied swine uterine flushings to luteinized granulosa cells in vitro. Uterine flushings on days 1 to 10 and day 20 of the estrous cycle produced no effect on the growth of granulosa cells or their progesterone production. Flushings obtained on days 14 to 18 of the estrous cycle were very luteolytic and had completely destroyed the cells within 6 to 8 hours. The luteolytic effect was also sometimes present in uterine flushings obtained on day 12 of the estrous cycle. Schomberg (1969) demonstrated that the uterine fluid component with luteolytic activity was thermolabile and nondialyzable, and the molecular weight was estimated to be about 200,000.

Mazer and Wright (1968) found a nondialyzable luteolytic factor in the uterus of the hamster on days 6 and 7 of pseudopregnancy. Lukaszewaska and Hansel (1970) found that extracts from bovine endometrium reduced hamster corpus luteum weight and progesterone content. The endometrial extracts were water soluble and precipitable in 55 percent ammonium sulfate. The 55 percent ammonium sulfate fraction had a partition coefficient of 0.271 when filtered through Sephadex G-100. The active factor was concluded to be either a large molecular weight protein or a smaller molecule bound to protein. Thus, the available

data relating to the endometrial luteolytic factor strongly suggest that it is proteinaceous.

Data from studies on embryo culture, tube-locked embryos, and delayed implantation suggest that embryos require some uterine factor in order to reach and surpass the expanding blastocyst stage of development. Experiments on superovulation and embryo superinduction have shown that in general (except in mice) polytocous species lack the ability to maintain litter sizes larger than those now characteristic of the species and that most of the embryonic death occurs early in gestation. Estrogens have been shown to stimulate DNA, RNA, and protein synthesis in the uterus and specific estrogen-induced proteins have been described. Also, a specific protein which stimulates expansion of blastocysts in vitro has been demonstrated in the uterine intraluminal fluid of rabbits during early pregnancy. These data seem adequate for formulation of the hypothesis that the uterus secretes a specific protein(s) which is necessary for blastocysts to expand and to implant.

Data are available to indicate that a protein(s) secreted by the endometrium is involved in the regression of the corpus luteum. In considering all these data together it is possible to hypothesize that the embryotrophic and luteolytic factors are the same protein(s), and in the absence of embryos to metabolize this protein(s), it exerts a lytic effect on the corpus luteum. This hypothesis would provide an explanation for estrous cycles in the nonpregnant female and absence of estrous cycles during pregnancy.

CHAPTER 2. EFFECTS ON DEVELOPMENT OF PORCINE EMBRYOS CAUSED BY RESTRICTION TO THE OVIDUCT

As indicated previously, many lines of evidence suggest that the uterus supplies the necessary factor(s) which allows continued development of the early embryo. Restriction of embryos to the oviducal environment allows development only to the early blastocyst stage in mice (Kirby, 1962 and Orsini and McLaren, 1967), rabbits (Westman *et al.*, 1931; Pincus and Kirsch, 1936; and Adams, 1958), rats (Alden, 1942), and sheep (Wintenberger-Torres, 1956).

This study was initiated for the purpose of establishing whether or not the uterus plays a similar essential role in early embryonic development in swine.

Materials and Methods

Ten crossbred gilts (nulliparous females, *Sus scrofa*) were used in this experiment. Intact boars were used at 12-hour intervals to check for estrous behavior in the gilts. The females were bred at 12 and 24 hours after onset of estrus, and surgery was performed 48 to 72 hours after onset of estrus. Anesthesia was induced with 5 percent sodium thiopental and maintained with methoxyflurane. The reproductive tracts were exposed by mid-ventral laporotomies, and one oviduct in each gilt was ligated at the tubo-uterine junction while the contralateral uterine horn was ligated about 10 cm posterior to the tubo-uterine junction and served as a control. The gilts were slaughtered either seven or eight days *post coitum*,

and the embryos present were recovered by flushing the oviducts and uteri with a physiological saline solution. Recovered embryos were observed under a stereomicroscope at a magnification of 10 X or 45 X to determine stage of development, and the diameter of the embryos was measured with an ocular micrometer. Gilts from which no embryos were recovered were considered nonpregnant and eliminated from the experiment. Data were analyzed by chi-square and the Student's t-Test (Steel and Torrie, 1960).

Results and Discussion

A total of 53 embryos were recovered from seven of the ten gilts and the data are summarized in Table 1. The difference between uterine and tube-locked embryos was striking. The tube-locked embryos were mostly fragmented and all remained within the zona pellucida while 83.8 percent of the recovered uterine blastocysts had emerged from the zona pellucida, formed an inner cell mass, and expanded to about three times the diameter of the zona pellucida (Plate 1). This difference in size of the embryos was highly significant ($P < 0.001$).

Recovery rates were based on the number of embryos recovered compared to the number of corpora lutea. The overall recovery rate from pregnant gilts was 58.9 percent. The rate of recovery of tube-locked embryos (45.7 percent) tended to be less than the recovery rate of uterine embryos (65.0 percent), but this difference was not statistically significant. The nonsignificant difference suggests that many of the tube-locked embryos may have already disintegrated by the time of recovery.

TABLE 1. COMPARISON OF DEVELOPMENT OF TUBE-LOCKED VERSUS UTERINE EMBRYOS IN GILTS

Days post coitum	Site of recovery	No. of oviducts or uteri	Corpora lutea, no.	Embryos recovered, no.	Embryo recovery, %	Stage of embryonic development	Embryo diameter, av., mm
7	Uterus	4*	26	13	50	blastocysts	0.22
7	Oviduct	2	14	6	43	fragmented, 4-cell to morula	0.18
8	Uterus	5*	31	24	77	blastocysts	0.60
8	Oviduct	3	19	10	63	fragmented, 4-cell to morula	0.17

*Includes one animal in which the oviduct ligation did not prevent passage of embryos into the uterus and all of the embryos developed to the blastocyst stage.

PLATE 1

Figure 1. A tube-locked embryo recovered from gilt 324 seven days post coitum X 150.

Figure 2. A uterine blastocyst recovered from gilt 324 seven days post coitum X 150.

Figure 3. A tube-locked embryo recovered from gilt 331 seven days post coitum X 150.

1

2

3

Since no blastocysts were recovered from ligated oviducts, it is apparent that pig embryos must reach the uterine environment in order for blastulation to occur as is true for mice, rabbits, rats, and sheep. This provides an even broader basis for the hypothesis that the uterus of the gilt may secrete a protein(s) required for continued embryonic development.

CHAPTER 3. QUANTITATIVE AND QUALITATIVE CHANGES IN PROTEIN SECRETION BY THE SWINE UTERUS DURING THE ESTROUS CYCLE

In the preceding chapter data are presented that demonstrate that pig embryos fail to reach the blastocyst stage *in vivo* if they are prevented from reaching the uterus. This, along with data presented by other workers on embryo culture, superovulation, and embryo superinduction, strongly suggests that some uterine factor is necessary for continued development of swine embryos through the blastocyst stage. In the rabbit, a protein component of uterine fluid has already been shown to stimulate blastocyst growth *in vitro* (Krishnan and Daniel, 1967). Therefore, the protein components of swine uterine fluid were studied to determine whether there are proteins which are specific to the uterus, and whether there is variation in uterine specific protein which might be related to embryonic development.

Materials and Methods

Sixty-six crossbred gilts served as uterine fluid donors. The females were checked for estrous activity twice daily at 12-hour intervals with mature boars. Gilts were subjected to cervical stimulation with an insemination rod at 12 and 24 hours after the onset of estrus. The day of the estrous cycle on which the uterus of a given gilt was to be flushed was randomly assigned prior to the experimental period. Surgery dates were assigned as multiples of 24 ± 6 hours after onset of estrus. The first day of estrus was designated as day 0. Animals were held off feed and water for 24 to 36 hours before surgery. At surgery anesthesia was

induced with 5 percent sodium thiopental and maintained with methoxyflurane. The reproductive tract was exposed by mid-ventral laporotomy. A small incision was made in an avascular area of the oviduct approximately 1 cm from the tubo-uterine junction and a polyvinyl catheter (I.D. = 1.25 mm) was inserted through this incision and the tubo-uterine junction into the lumen of the uterus. While the external uterine bifurcation was being restricted with hand pressure, 20 ml of 0.33 M NaCl was injected into each uterine horn via the cannula. The saline was forced to the external bifurcation of the uterus by pulling the uterus between the thumb and forefinger; then it was forced back and out through the cannula into a sterile serum bottle. Flushings from the two horns were pooled and kept in an ice bath until centrifugation and sterilization could be carried out. The flushings were centrifuged at 3000 g and sterilized by filtration through 0.2 μ millipore filters. The sterilized flushings were then stored at -70°C until they could be analyzed.

The uterine flushings of approximately 40 ml were concentrated to about 1 ml by pressure dialysis. The fluid was then dialyzed against 0.05 M phosphate-citrate buffer, pH 7.4, for 24 to 36 hours. The protein concentration of each sample was determined by the method of Lowry et al. (1951), and the total quantity of protein in each sample was then calculated. These data were used to compare the relative amounts of total protein per uterus for each day of the cycle. This comparison depends upon the assumption that the same proportion of intraluminal fluid was recovered and the same proportion of the protein in the uterine flushings was lost during processing, regardless of the day of the estrous cycle.

Whole dialyzed samples, remaining after the protein determination and polyacrylamide disc electrophoresis, or aliquots of samples were

loaded on columns of Sephadex G-200. For most samples 1.5 x 90 cm columns were used and the bed heights varied from 75 to 85 cm. Other samples were loaded onto a 2.5 x 100 cm column. The eluent used was 0.05 M phosphate-citrate buffer, pH 7.4, and the flow rate was approximately 4 ml per hour for the small columns and 8 ml per hour for the large column. Gel filtration was carried out at 3°C. The fractions were approximately 2 and 4 ml in the small and large columns, respectively. Aliquots of each fraction were used for protein determination. The optical density of each fraction was measured and plotted against the elution volume at which the fraction occurred, so that a protein profile was constructed.

It was impossible to load a standard quantity of protein from each sample onto the column; therefore, the optical densities obtained from the fractions of one sample were not directly comparable to those from other samples. In order to standardize all gel filtration data, the optical density of each fraction was converted to a percentage of the total protein eluted from the column for each sample. This allowed the construction of protein profiles which were directly comparable to each other. Thus, protein profiles of samples within and among days of the estrous cycle were compared.

Polyacrylamide gel disc electrophoresis was employed for further characterization of the proteins in the uterine flushings during the various days of the estrous cycle. The electrophoresis was carried out in 7 percent acrylamide gel; however, neither sample gel nor spacer gel was used. Instead, the samples were applied in 1 M sucrose. Electrophoresis was carried out at 2.2 mA. per gel in 0.05 M Tris-0.38 M glycine

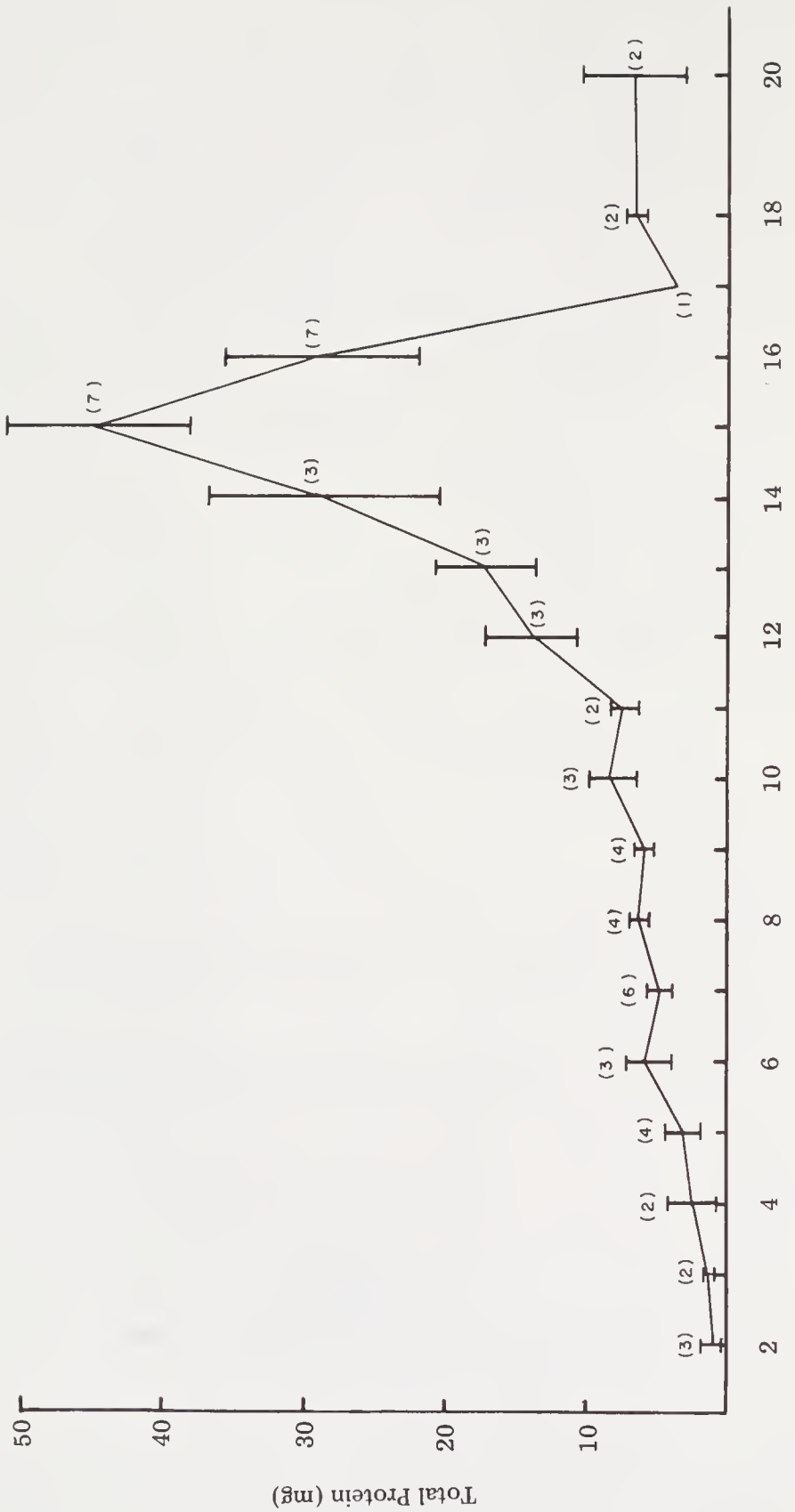
buffer, adjusted to pH 8.0 by addition of HCl. Gels were stained with amido black or periodic acid-Schiff (PAS) and then destained. The gels were then compared directly or indirectly from densitometer tracings.

Results and Discussion

One of the samples of uterine flushings obtained on day 3 and one obtained on day 6 were excluded from the results due to the presence of excessive amounts of blood and hemoglobin. A sample of day 4 and one of day 7 flushings were lost during dialysis due to defective dialysis tubing, and a sample of day 18 flushings was not used since the animal from which it was obtained exhibited one estrous cycle that was abnormally long (24 days).

The mean values for total protein flushed from uteri on each day of the estrous cycle and the standard error of each mean are presented in Figure 4. This shows clearly that the protein content of uterine secretion remained fairly stable from day 2 through day 9 of the cycle and then it began to increase. The increase became sharper on day 13 and the maximum value was reached on day 15, after which the total protein content of uterine flushings decreased sharply so that on days 18 and 20, the amount was comparable to that on days 6 to 9 of the cycle. The peak in protein secretion occurred while the reproductive system was under the influence of progesterone, suggesting that progesterone, possibly in synergism with estrogen, is responsible for the increased production of protein. Also the abrupt decrease in the protein content of uterine flushings coincided with the regression of the corpora lutea (Masuda *et al.*, 1967) and reduced progesterone production in the pig (Gomes *et al.*, 1965;

Figure 4. Variation in the total quantity of swine uterine fluid protein secreted per day of the estrous cycle. The number of samples (numbers in parentheses) contributing to the respective means and the standard error of each mean (vertical lines) are indicated at each point in the graph.


Total Protein (mg)
50
40
30
20
10
2
4
6
8
10
12
14
16
18
20
Day of Estrous Cycle
(3)
(2)
(2)
(4)
(3)
(6)
(4)
(4)
(2)
(3)
(3)
(3)
(7)
(7)
(1)
(2)
(2)

Schomberg et al., 1966; and Masuda et al., 1967). This further suggests that progesterone plays a role in the secretion of protein by the uterus of the pig.

Protein profiles of uterine flushings obtained on days 2 through 18 and day 20 are presented in Figure 5. From these profiles it can be seen that all profiles contain three protein fractions which do not appear to vary with days of the estrous cycle. These three fractions (Fractions I, II, and III) have estimated average molecular weights of 400,000 and greater, 200,000 and 90,000, respectively. Beginning on day 9 and continuing through day 16, an additional peak (Fraction V) with an average molecular weight of 20,000 occurred in the protein profile. Fraction V constituted greater than 20 percent of the total protein on days 12 through 16, although it is quantitatively greatest on day 15.

Polyacrylamide gel disc electrophoresis revealed that Fraction V consists of six acidic proteins (Plate 2, Figures 6a and b). Although most, if not all, of these six proteins were absent from flushings obtained prior to day 9 and after day 16, it is possible that they may have been present in undetectable quantities prior to day 9. When the portion of the gel filtration eluent which contained Fraction V was compared across all the days of the cycle, a slight increase occurred on days 5 and 6 (see Figure 7). It is not known whether this transitory increase is physiologically significant; however, it would seem that any uterine secretion critical for the development of the blastocyst must be present at the time when this stage is reached.

On days 12 through 16, the region between Fraction III and Fraction V of the gel filtration protein profile was occupied by a lavender protein with a molecular weight of about 45,000, as determined by gel filtration.

Figure 5. Variation in gel filtration protein profiles with days of the estrous cycle.

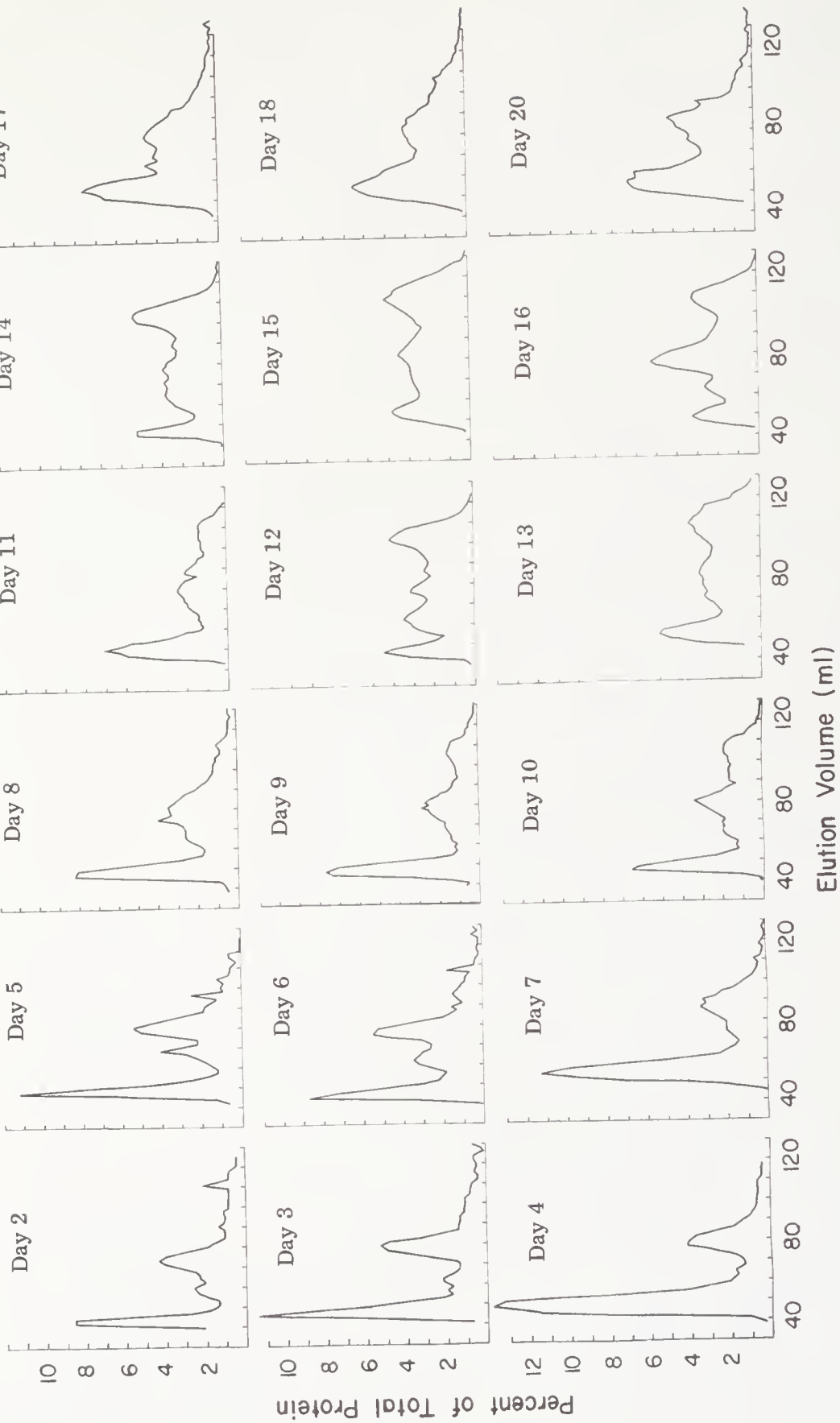
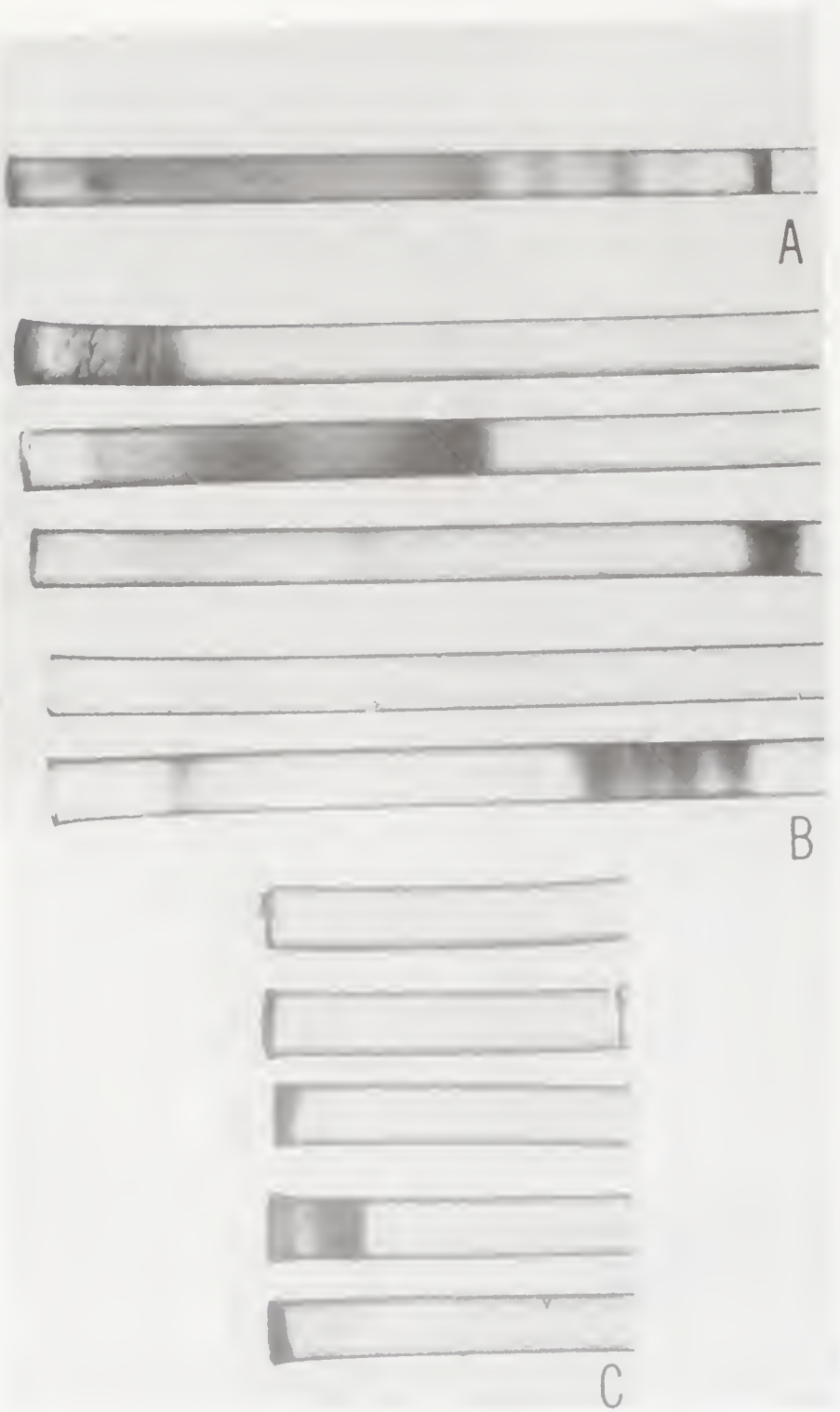

Day 2
Day 5
Day 8
Day 11
Day 14
Day 17
Day 3
Day 6
Day 9
Day 12
Day 15
Day 18
Day 4
Day 7
Day 10
Day 13
Day 16
Day 20
Percent of Total Protein
Elution Volume (ml)

PLATE 2

Figure 6a. Polyacrylamide gel disc electrophoresis of swine day 15 uterine fluid proteins, migration toward the anode (from left to right).

Figure 6b. Polyacrylamide gel disc electrophoresis of Fractions I (top of figure) through V (bottom of figure), migration toward the anode (from left to right).

Figure 6c. Polyacrylamide gel disc electrophoresis of Fractions I (top of figure) through V (bottom of figure), migration toward the cathode (from left to right).


A
B
C

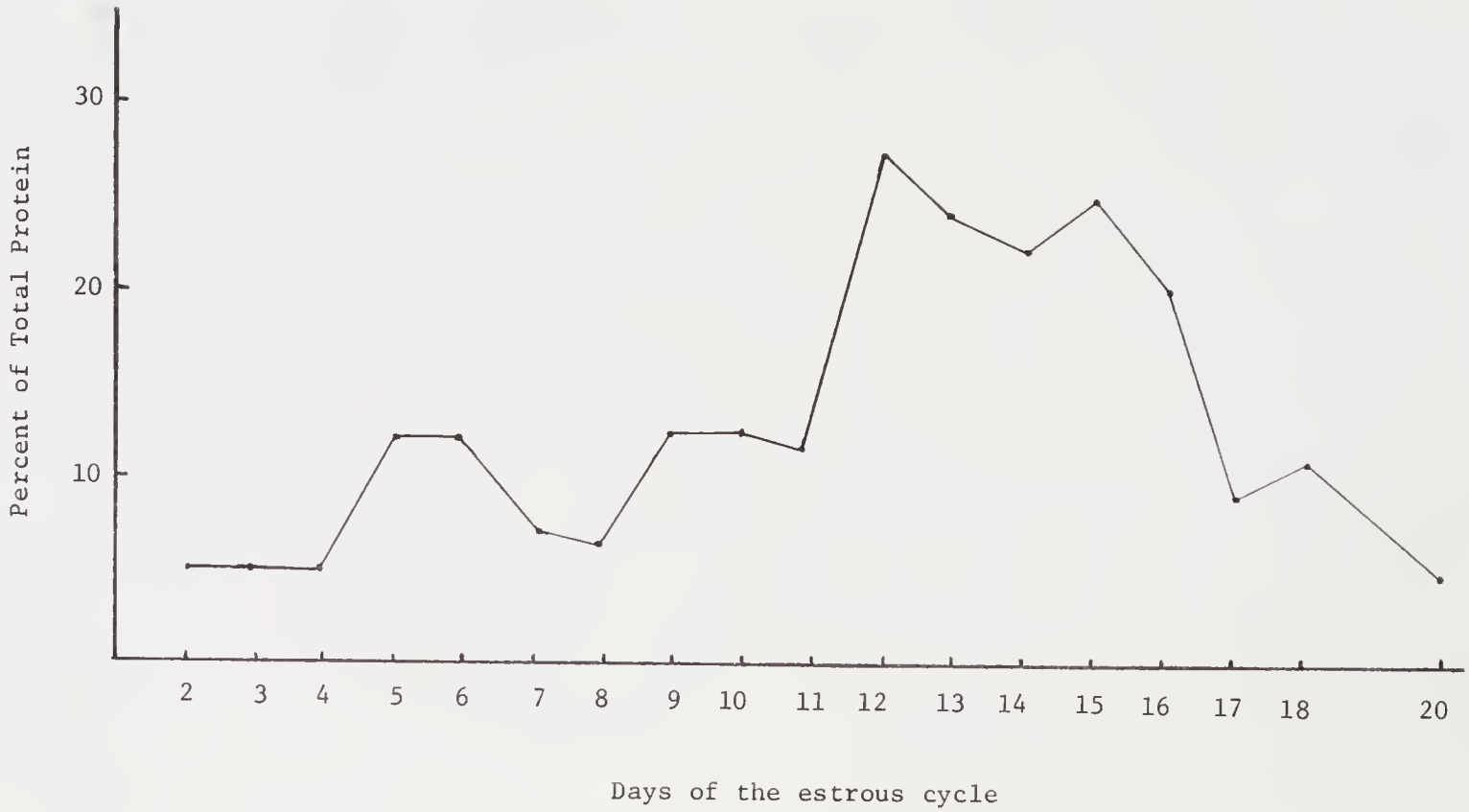


Figure 7. Variation with days of the estrous cycle in the relative percentage of uterine fluid proteins with molecular weight estimates of 10,000 to 25,000.

The lavender material was stained by both amido black and PAS. In addition to the lavender protein, polyacrylamide gel disc electrophoresis demonstrated that two other proteins may be included in Fraction IV (Plate 2, Figure 6c). All three of the proteins migrated toward the cathode at pH 8.0, and Fraction IV contained no protein which migrated toward the anode at this pH. The secretion pattern of Fraction IV proteins, as is the case with Fraction V proteins, suggests that if any role in embryonic development is fulfilled by these molecules, it must relate to the growth of the expanding blastocyst rather than the process of blastulation.

The pattern of secretion of both Fractions IV and V proteins implies that the presence of a functional corpus luteum is necessary for their secretion. There was a rather gradual increase in the amounts of these proteins until their peak was reached on day 15. At this point the corpus luteum in the pig has reached an advanced state of regression and lost much of its secretory function (Gomes et al., 1965; Schomberg et al., 1966; and Masuda et al., 1967), and the secretion of Fractions IV and V decreased abruptly. The data also support the hypothesis that uterine proteins are responsible for regression of the corpus luteum, as is suggested by the data of Schomberg (1967), Mazer and Wright (1968), and Lukaszewaska and Hansel (1970). However, the secretion patterns of Fractions IV and V do not suggest a role for them in the process of blastulation and blastocyst expansion as is true of the "blastokinin" secretion pattern in the rabbit (Krishnan and Daniel, 1967 and 1968; Beier, 1968; and Kirchner, 1969). It is possible that the amount of stimulatory proteins needed for blastulation may be very small and therefore undetectable by the methods used in this study. If this is true, the transitory

increase in the low molecular weight fraction on days 5 and 6 may be related to early embryonic development. On the other hand, Fractions IV and V may be related to the rapid growth and expansion of the more advanced embryo.

CHAPTER 4. PREGNANCY IN GILTS AFTER INTRAVENOUS INJECTIONS OF ANTI-PORCINE UTERINE FLUID PROTEINS

Evidence was presented in the preceding chapter that two groups of proteins specific to the uterus are secreted from day 9 to day 16 of the estrous cycle of the pig. Some possible roles for these proteins in reproduction were also discussed. This experiment was designed to determine the effect of passive immunization of gilts against uterine fluid proteins.

Materials and Methods

Day 14 is approximately midway through the phase of the estrous cycle in which major quantitative and qualitative changes occur in the swine uterine fluid protein secretion pattern. Therefore, uterine proteins obtained on day 14 of the estrous cycle were used in this experiment. Uterine fluid was obtained, prepared, and chromatographed through Sephadex G-200 as described in Chapter 3.

Sheep were used to produce antibodies against Fractions I through V (anti-total) of swine uterine proteins, Fractions I, II, and III (anti-high) of swine uterine proteins, Fractions IV and V (anti-low) of swine uterine proteins, and Freund's complete adjuvant (anti-Freund). Uterine proteins in doses of 2 - 16 mg in suspensions of Freund's complete adjuvant were injected intradermally into the sheep, and these doses were administered twice for Fractions I to V and for Fractions I, II, and III, but three times for Fractions IV and V. In each case all of the antigens

available at the time of the injections were used. Totals of 5.7 mg of Fractions I to V; 32.0 mg of Fractions I, II, and III; and 6.9 mg of Fractions IV and V were administered to the appropriate sheep. The sheep were challenged with the respective antigens at intervals of two weeks and harvesting of antisera was begun four weeks after the initial challenge. A commercial preparation of goat anti-porcine serum was used for comparison with the sheep anti-porcine uterine protein sera prepared for this experiment. Specificities of the antisera were determined by comparing the anti-porcine serum with the anti-porcine uterine protein sera using diffusion-in-gel and immunoelectrophoresis. The antisera were stored at -20°C.

Treatment of Gilts - Trial I

Five mated gilts received injections of antisera via an ear vein. Two gilts were injected with anti-total sera, two with anti-low sera, and one with anti-Freund sera. Each gilt received 5 ml of the appropriate antiserum on days 8 and 10 post coitum and 10 ml of the proper antiserum on days 12 and 14. The gilts were laporotomized on day 30 after mating and their reproductive tracts were examined for pregnancy and condition of corpora lutea.

Treatment of Gilts - Trial II

Eleven gilts were used in Trial II. Six gilts were mated to fertile boars and five gilts were nonmated. One nonmated and two mated gilts received 10, 15, and 20 ml of anti-high serum on days 5, 7, and 9 after the onset of estrus, respectively. Two mated and nonmated gilts received the same amounts of anti-low on days 5, 7, and 9. Two mated and two nonmated gilts were treated with 50, 100,

and 200 ml of anti-low on days 10, 12, and 14 after the onset of estrus. Using techniques described in Chapter 3, uterine fluid was obtained from all nonmated gilts on day 15 of the estrous cycle. The fluids were processed in the manner previously described and each sample was subjected to gel filtration. Mated gilts were laporotomized on day 30 post coitum and their reproductive tracts were examined for pregnancy and condition of corpora lutea.

Results and Discussion

Immunoelectrophoresis revealed four antibodies in anti-low serum which cross-reacted with basic proteins in rechromatographed Fraction IV. Two diffuse precipitation crescents were evident after immunoelectrophoresis of rechromatographed Fraction V. None of these six antibodies was present in anti-porcine serum, although at least three antigens in total uterine fluid cross-reacted with anti-total, anti-high, and anti-porcine sera. Anti-high serum contained at least six antibodies not found in anti-porcine serum and anti-total contained a combination of the antibodies found in anti-high and anti-low sera. Thus, at least 12 antigenic substances, apparently unique to the secretions of the uterus, were identified using immunoelectrophoresis. Anti-Freund serum did not cross-react with any antigen in pig uterine flushings.

After intravenous injections of sheep anti-swine uterine protein sera into mated pigs, no effects were apparent at laporotomy 30 days after mating in either Trial I or Trial II. Possible reasons for the lack of effect include lack of antibodies against specific proteins required by the embryos, failure of antibodies to reach the uterine lumen, inadequate quantities of antibodies, and lack of a requirement by swine embryos for specific uterine fluid protein.

There was also no apparent effect of antisera on the total protein content or gel filtration protein profile of uterine flushings obtained from the nonmated gilts. The total quantity of protein obtained from the uterine fluid of these animals was well within the range of values for total protein in other samples of day 15 uterine fluid described in Chapter 3. The failure of these antisera to affect the protein constituents of uterine flushings suggests that the antibodies did not reach the uterine lumen. Also, estrous cycle lengths were normal following antisera treatments of nonmated gilts. Therefore, there was evidently no effect on the corpora lutea.

Although this experiment did not provide data on the relationship of uterine fluid proteins to embryonic development or to corpus luteum regression, it did provide valuable information. In this study it was learned that at least 15 different antigenic substances constitute the macromolecular fraction of swine uterine secretions, and that there are at least 12 antigens of uterine fluid which do not cross-react with anti-porcine serum.

CHAPTER 5. GENERAL DISCUSSION

A variety of studies has provided information regarding the influence of uterine factors on the early development of the mammalian embryo and, equally important, on the function of the uterus in the regression of the corpus luteum. The data obtained in this research provide additional information and support the concept that secretions of the uterus control embryonic development and the status of the corpus luteum.

In this experiment pig embryos failed to reach the blastocyst stage of development when prevented from reaching the uterus by ligation of the oviduct. In the mouse, rabbit, rat, and sheep a similar developmental failure of tube-locked embryos has been reported (Westman et al., 1931; Pincus and Kirsch, 1936; Alden, 1942; Wintenberger-Torres, 1956; Adams, 1958; Kirby, 1962; and Orsini and McLaren, 1967); however, in these species development to the early blastocyst stage in the oviduct was observed. These data lead to the unavoidable conclusion that the uterus provides some factor which is absolutely required for continuous development of the early embryo.

It does not appear likely that simple nutrients constitute the uterine factors which enable the uterine embryos to develop continuously. A wide variety of culture media have been used for in vitro cultivation of ova from a number of species of mammals to the blastocyst stage (Chang, 1949; Hammond, 1949; Whitten, 1956 and 1957; McLaren and Biggers, 1958; Tarkowski, 1961; Brinster, 1963; Adams, 1965; Cole and Paul, 1965; Onuma et al., 1968; Maurer et al., 1969; and Rundell, 1969). In these

studies the embryos underwent cleavage *in vitro* in synthetic media containing simple carbohydrates, inorganic salts, and sources of fixed nitrogen or in heated sera; yet development did not continue past the early blastocyst stage. These data indicate that simple nutrients are not adequate to support development through this critical phase.

An opposing view is held by two groups of workers. Mouse zygotes (Whitten and Biggers, 1968) and 2- to 4-cell rabbit embryos were grown to the expanding blastocyst stage *in vitro*. Possible reconciliation between these results and data indicating that embryos fail to continue development in the absence of the uterine environment is found in the work of Nicholas (1942), Fawcett *et al.* (1947), Runner (1947), Fawcett (1950), and Kirby (1962), which indicates that trophoblasts of transplanted oviducal embryos expand in a morphologically normal manner, but the embryonic disc cells failed to develop.

Once the embryo has surpassed the critical period of development, which appears to require the uterine environment, growth and development can again take place *in vitro* in the New (1967) culture medium recirculator. Hamster embryos (Givelber and DiPaolo, 1968), rabbit embryos (Daniel, 1970a), and rat embryos (New, 1967; and New and Daniel, 1969) have been so cultured. This information is important because it indicates that, if the embryo is allowed access to the uterine environment during the critical phase of differentiation, the embryo can be cultured *in vitro* during the period of organogenesis.

Evidence that the uterus exerts control over the embryo has been obtained from studies involving embryo superinduction, superovulation, asynchrony of embryonic and uterine development, and delayed implantation.

In a series of experiments it was shown that embryo superinduction of gilts with 2.5-day embryos did not affect litter size at 105 days, 90 days, or 25 days after mating (Bazer et al., 1969 a,b). Fenton et al. (1969a) reported that 7-day embryos used for superinduction of gilts increased litter size at 25 days post coitum. This led to the hypothesis that the uterine control of embryonic development is exerted prior to day 7 post coitum. To test this hypothesis 2.5-day and 7-day embryos were used for embryo superinduction (Fenton et al., 1969 b), but in this experiment 7-day embryos did not result in an increase in litter size as compared to unoperated controls or gilts superinducted with 2.5-day embryos. Schwartz et al. (1970) obtained similar results after embryo superinduction with 7-day embryos. These data suggest that the uterine control of embryonic survival is exerted after day 7 but before day 25 post coitum.

The effect of reduced uterine space on embryonic development was studied by Dziuk (1968), Fenton et al. (1970), and Johnson (1970). In gilts (Dziuk, 1968; and Fenton et al., 1970) the reduction of uterine space had no effect on litter size at 25 days after mating although 105-day litter size was reduced. In mice, rabbits, and rats (Johnson, 1970) one uterine horn was found to carry as many embryos to term as two uterine horns. These data suggest that some factor other than uterine space limits litter size since the majority of the embryonic deaths occur prior to the time when uterine space becomes limiting.

Superovulation of the pig (Hammond, 1921; Perry, 1954; King and Young, 1957; Rathnasabapathy et al., 1957; Hunter, 1966; Longenecker and Day, 1968; Pope et al., 1968; and Bazer et al., 1969 c) and the rabbit (Adams, 1960; Hafez, 1964; and Johnson, 1970) did not result in

increased litter size. On the other hand, litter size was increased by superovulation of mice (McLaren and Michie, 1956; McLaren and Michie, 1959 a,b; and Wilson and Edwards, 1963), except in the experiment of Fowler and Edwards (1957). In general, litter size cannot be increased by embryo superinduction or by superovulation in polytocous species. This suggests that there is a limit to the number of embryos that can be maintained ("uterine capacity"), and that this limit is closely approached during normal pregnancy in pigs and rabbits.

Embryonic survival is greatest when the transferred embryos and recipient uteri are closely synchronized (Bazer et al., 1969a; Dickmann and Noyes, 1960; and Hunter et al., 1967). Furthermore, Bazer et al. (1969a) showed that when transferred embryos and recipient uteri were in asynchrony, the embryos native to the recipient uteri had significantly higher survival rates than transferred embryos; whereas, there was no statistical difference in survival rates if the transfer was made in synchrony. From this it can be inferred that the uterus secretes some substance or a group of substances which are necessary for embryonic development, that the substance(s) is produced at a specific time, and that the embryo must be present in the uterus at the proper time in order to utilize the secretion.

Further information on the relationship between the uterus and embryonic development is available from experiments with animals exhibiting delayed implantation (Daniel, 1970b). Dickmann and DeFeo (1967) demonstrated that the uterine development rather than the embryo determines whether or not implantation in the rat is to be delayed. This also supports the idea that uterine secretions are necessary for continuous embryonic development and that the secretion of the required

material is not constant but instead is produced at a specific time.

Because of their interesting biological functions as enzymes and regulators of genetic material, proteins are worthy of study as possible uterine specific substances required by embryos in order for differentiation to proceed in a normal manner. Studies based on electrophoresis (Junge and Blandau, 1958; Ringler, 1961; Albers and Castro, 1961; and Stevens *et al.*, 1964) led to the conclusion that intraluminal fluids from rat and rabbit uteri contain proteins similar to serum proteins as well as proteins specific to the uterus.

Krishnan and Daniel (1967) were the first investigators to show that a specific uterine protein is secreted in large quantities at a precise time in early pregnancy in the rabbit, and that this protein can influence a definite phase in the development of the embryo *in vitro*. The protein, designated as "blastokinin," was shown to appear on day 3 *post coitum*, reach a maximum concentration on day 5, and then decline. "Blastokinin" was found to stimulate blastulation *in vitro*. Other workers (Beier, 1968; Kirchner, 1969; and Hamana and Hafez, 1970) have also demonstrated the same protein or a very similar protein in rabbits.

In the present experiment the proteins in swine uterine flushings were studied on days 2 through 18 and day 20 of the estrous cycle. Gel filtration of the proteins in these flushings demonstrated that at least two groups of proteins were not secreted constantly throughout the estrous cycle, but instead appeared at mid-cycle and disappeared before the next estrus. The fraction (Fraction IV) having the higher molecular weight (45,000) contained at least three proteins as revealed by polyacrylamide gel disc electrophoresis. All three

proteins are basic at pH 8.0. The remaining protein fraction (Fraction V) had an estimated molecular weight of approximately 20,000. Polyacrylamide gel disc electrophoresis demonstrated six acidic proteins and one slightly basic protein in Fraction V at pH 8.0. Thus a total of at least 10 proteins with molecular weights between 10,000 and 50,000 are secreted in a cyclic pattern by the uterus. Proteins with higher molecular weights may also be secreted at specific periods during the estrous cycle; however, the methods used in this study were not adequate to permit drawing such a conclusion.

Since all the proteins in Fraction IV and one in Fraction V of gilt uterine fluid are basic at pH 8.0, they are well suited as repressors or inducers, and therefore they are possibly regulatory in function. Induction of regulatory protein synthesis by the uterus may be an important function of estrogens and progesterone. Estrogen (Hamilton, 1963; Ui and Mueller, 1963; Hamilton, 1964; Gorski and Nelson, 1965; Notides and Gorski, 1966; Hamilton et al., 1967; and Billing et al., 1969) and progesterone (Dingman et al., 1969; and O'Malley and McGuire, 1969) have been shown to stimulate RNA and protein synthesis in mammalian uterine and chick oviduct tissues. Proteins induced by these hormones may possibly serve regulatory functions in the uterus and/or in embryos.

Prasad et al. (1968) found that only very low rates of DNA, RNA, and protein synthesis occur in rat uteri and blastocysts undergoing delayed implantation; however, estrogen stimulated DNA, RNA, and protein synthesis in the inner cell mass of the blastocysts and RNA and protein synthesis in the uterus. These data indicate

that estrogen can initiate a resumption of growth and metabolic activity in diapausing rat embryos, apparently by induction of protein synthesis. If the basic proteins observed in the present study serve as repressors or inducers of specific regions of the DNA of swine embryos, they should offer excellent opportunities for the study of basic mechanisms of embryonic differentiation.

An attempt to determine biological functions of the protein fractions obtained in the present study has been carried out. Immunization of gilts against uterine fluid proteins did not affect the embryos, corpora lutea, or uterine protein secretions in any apparent way. The gilts may have received insufficient doses of the antisera, or the antibodies may not have reached the antigens. Use of larger quantities of antisera and early administration directly into the uterine lumen might be advantageous in a future experiment. Injections of antisera into gilts did not provide additional data on the relationship between uterine proteins and embryonic development or corpus luteum regression. However, valuable information on the specificity of the uterine protein secretion was obtained.

By use of the techniques of diffusion-in-gel and immunoelectrophoresis, it was learned that at least 12 proteins, not found in pig serum, are secreted by the uterus of the pig and of these, six occurred in Fractions IV and V. Three additional uterine proteins are also present in pig serum. These data indicate that the uterine fluids used in this research represent true secretions of the uterus.

Recently the effects of uterine fluid proteins on mouse embryonic development were tested (unpublished data, Ulberg et al., 1970). In this experiment 4- to 8-cell mouse embryos were cultured in Brinster's medium for ova culture (Brinster, 1963), supplemented with total uterine fluid protein or fractions of the total. The fractions used were those described in Chapter 3. The supplements were added in concentrations of 1 mg/ml or 0.1 mg/ml. The embryos were cultured in these media for up to 96 hours. During this time most embryos (82.0 percent) reached the blastocyst stage and 27.2 percent hatched. As evaluated by percentage of embryos which hatched and embryonic size, no fraction was as stimulatory to mouse embryonic development in vitro as was the total of all uterine proteins. However, the percent of embryos hatching in media containing Fractions I, II, IV, and V in concentrations of 0.1 mg/ml was superior to that in concentrations of 1 mg/ml. The percent of embryos hatching in media containing Fraction III was about the same at 0.1 mg/ml and 1 mg/ml. On the other hand, embryonic diameter tended to be smaller when embryos were grown in media containing uterine protein at 0.1 mg/ml than at concentrations of 1 mg/ml. The data suggest that more than a single protein may be required for optimum embryonic development, since no single fraction was as stimulatory as the total of all proteins. For this reason, it would be informative to test the effects of all possible combinations of the five fractions on swine embryos in vitro.

Possible luteolytic effects of the proteins in swine uterine flushings were not tested in the present study; however, there is ample evidence that uterine proteins are luteolytic (Schomberg, 1967,

1969; Williams et al., 1967 a,b; Mazer and Wright, 1968; and Lukaszewaska and Hansel, 1970). Therefore it would be useful to know which fraction(s) of the swine uterine flushings is luteolytic in order to gain more information concerning interrelationships among the uterus, corpus luteum, and embryo. It is very interesting that Fraction IV proteins and Fraction V proteins were observed to be present in highest concentrations in swine uterine flushings at the same time in the estrous cycle that Schomberg (1967) found swine uterine flushings to be luteolytic.

After consideration of the several lines of evidence which suggest that the uterus plays an active role in mammalian reproduction, the implications were integrated to form a hypothesis concerning the active role of the uterus. The hypothesis assumes that the uterus secretes a protein(s) which is required by the embryo for development; and if no embryos are present to utilize the protein(s), it causes regression of the corpus luteum and the estrous cycle begins anew. The information gained in the present study is inadequate to allow conclusions to be drawn concerning the validity of this hypothesis; however, the data obtained provide a basis for further experiments to test the hypothesis.

SUMMARY

The uterus actively influences mammalian reproduction by participation in embryonic differentiation and in determination of estrous cycle length. In these experiments the secretion of proteins by the porcine uterus during the estrous cycle was characterized and related to reproductive phenomena.

A preliminary experiment was designed to determine if the porcine uterus provides an environment essential for continued development of the early embryo. One tubo-uterine junction was ligated in each of 10 mated gilts under surgical anesthesia at 48 to 72 hours post coitum. The contralateral uterine horn was ligated approximately 10 cm posterior to the tubo-uterine junction and served as the control side. The animals were slaughtered at either 7 or 8 days after mating and the embryos were recovered. The tube-locked embryos failed to develop past the morula stage and many had begun degenerating by the time of recovery. No tube-locked embryo had escaped from the zona pellucida. On the other hand, uterine embryos in the same females were mostly blastocysts, and 84 percent had emerged from the zona pellucida, formed a distinct inner cell mass, and expanded to approximately three times the diameter of the zona pellucida. Thus it was shown that developmental failure occurred in embryos prevented from reaching the environment provided by the uterus.

The daily variation in the protein content of porcine uterine intraluminal fluid was studied on days 2 to 18 and day 20 of the estrous cycle. Sixty-six gilts furnished one sample of uterine fluid each. The

samples were centrifuged at 3000 g, sterilized by filtration, concentrated from approximately 40 ml to 1 ml, and dialyzed against 0.05 M phosphate-citrate buffer, pH 7.4. A portion of each sample was chromatographed through Sephadex G-200 and another portion was subjected to polyacrylamide gel disc electrophoresis. Gel filtration through Sephadex revealed three groups of proteins (Fractions I, II, and III) which did not vary with the estrous cycle and two groups of proteins (Fractions IV and V) which were not present early in the estrous cycle. Fraction IV, which appeared on day 12, contained a lavender protein with an estimated molecular weight of 45,000. Fraction V had an estimated molecular weight of about 20,000 and appeared as early as day 9. Fractions IV and V were not present in the gel filtration protein profiles after day 16 of the cycle.

Polyacrylamide gel disc electrophoresis demonstrated that Fraction IV contained at least three basic proteins, while Fraction V contained one basic protein and six acidic proteins at pH 8.0. The basic proteins, especially, are interesting as possible regulators of the differentiation of embryos by control of genetic expression. Also, these proteins appear in the uterine flushings shortly before the corpus luteum begins to regress and are not present after regression. This coincidence suggests an interrelationship between the low molecular weight proteins and the corpus luteum.

Attempts to determine the role of the uterine fluid proteins in reproductive phenomena have been largely unsuccessful. Intravenous injections of anti-uterine fluid sera did not interfere with pregnancy in mated gilts or cause any quantitative or qualitative change in the

protein content of uterine fluid from nonmated gilts. However, it is not possible to draw final conclusions from these data, since there is a possibility that the antibodies injected did not reach the uterine lumen in sufficient quantities to bind the proteins secreted by the uterus.

This study showed that the uterine environment is required for embryonic development and that there are cyclic changes in the protein secretion pattern of the uterus. These findings provide additional support for the concept that the proteins of maternal origin may regulate genetic expression in the embryo during early differentiation.

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BIOGRAPHICAL SKETCH

Finnie Ardrey Murray, Jr. was born May 30, 1943, at Burgaw, North Carolina. In May, 1961, he was graduated from Burgaw High School. During the summer of 1963, he served in Operation Crossroads, Africa and worked in Serowe, Botswana. From 1964 until 1965 he worked as a laboratory technician in the Dairy Science Department of North Carolina State University. In June, 1966, he received the degree of Bachelor of Science with majors in Animal Science and Zoology from North Carolina State University. In 1966 he enrolled in the Graduate School of North Carolina State University. He worked as a graduate research assistant in the Department of Animal Science until September, 1968, when he received the degree of Master of Science with a major in Physiology. In 1968 he enrolled in the Graduate School of the University of Florida and worked as a graduate research assistant until October, 1970. From September, 1968, until the present time he has pursued his work toward the degree of Doctor of Philosophy.

Finnie Ardrey Murray, Jr. is married to the former Margaret Alice Kelly and is the father of a son, Michael Edward. He is a member of Alpha Zeta, the American Society of Animal Science, Phi Sigma, Sigma Xi, and the Society for the Study of Reproduction.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Alvin C. Warnick, Chairman
Professor of Animal Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Fuller W. Bazer
Assistant Professor of Animal Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

James A. Himes
Assistant Professor of Veterinary Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ray L. Shirley

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This dissertation was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

March, 1971

Dean, College of Agriculture

Dean, Graduate School